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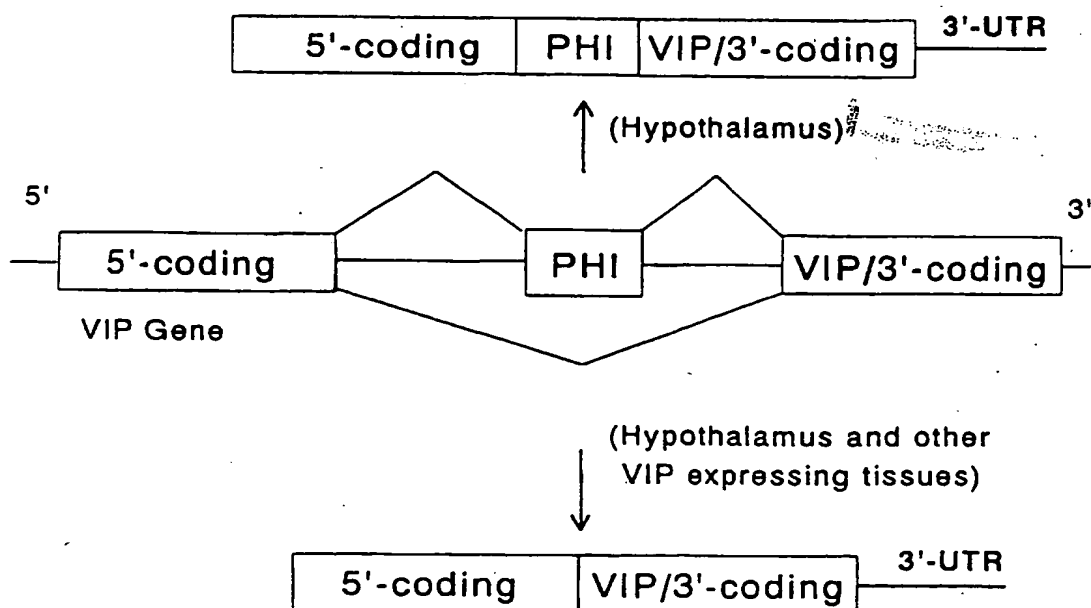
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(54) Title: DNA ENCODING TURKEY HYPOTHALAMIC VASOACTIVE INTESTINAL PEPTIDE



(57) Abstract

The present invention provides an isolated and purified DNA molecule comprising a single coding region encoding (a) a turkey vasoactive intestinal peptide (VIP); (b) a turkey prepro vasoactive intestinal peptide or (c) a biologically active subunit of (a) or (b).

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DNA ENCODING TURKEY HYPOTHALAMIC  
VASOACTIVE INTESTINAL PEPTIDE

5

Background of the Invention

The avian hypothalamus exerts a principally stimulatory influence  
10 on prolactin (PRL) secretion. Several lines of evidence support vasoactive  
intestinal peptide (VIP) as the most important prolactin-releasing factor (PRF) in  
birds. One vasoactive intestinal octacosapeptide is produced naturally in chickens  
and may be referred to as chicken VIP or cVIP. This particular VIP has the  
amino acid chain of His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Ser-Arg-Phe-  
15 Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Val-Leu-Thr. cVIP is  
cross-reactive with any bird of the Avian species, including turkeys and ducks.  
In other words, turkeys and ducks also recognize cVIP.

VIP meets many of the qualifications of a PRF. For example, VIP  
stimulates PRL release from the anterior pituitary *in vitro*, and the response is  
20 closely correlated to the reproductive state of the animal. VIP also stimulates  
PRL release *in vivo* in median eminence-deafferentated hens (see, Opel et al.,  
Proc. Soc. Exp. Biol. Med., 187, 455 (1988)). Also, L.J. Mauro et al., Gen.  
Comp. Endoc., 87, 481 (1992) reported the presence of high VIP concentration in  
median eminence, particularly the external layer. The presence of high affinity  
25 VIP receptors on the anterior pituitary cells has also been reported.

Further evidence that VIP is a PRF is provided by recent findings  
that lesioning of VIP cell bodies in the infundibular nuclear complex (INF)  
eliminates PRL increases associated with the photo-induced reproductive cycle  
and suppresses elevated PRL associated with incubation behavior. Finally,  
30 immunoneutralization of turkeys with endogenous VIP reduced both circulating  
PRL and pituitary PRL mRNA, totally blocked the PRL release effected by

electrical stimulation of the medial preoptic nucleus, and blocks the hormonal and behavioral characteristics of incubating hens.

For example, the active immunization of turkey hens with VIP conjugated to an adjuvant protein was found to increase egg production.

5 Although the bio-mechanical mechanism is not fully understood, it is believed that the antibodies so produced complex the turkey hen's naturally produced VIP. This naturally produced VIP regulates the secretion of the hormone prolactin from the pituitary gland of the turkey hen. In turn, increased prolactin secretion causes broodiness in turkey hens. Broodiness, in turn, is one of the factors that  
10 may lead to poor egg production. Accordingly, egg production is enhanced by binding the turkey hen's naturally produced VIP with the natural antibodies generated by the turkey before the turkey's VIP can act upon the turkey's pituitary gland to increase prolactin secretion.

Because of the prominent role VIP plays in the regulation of PRL  
15 secretion, it is likely that the secretion of PRL is mediated through changes in VIP secretion and/or gene expression, which may vary between the hypothalamic and hyperlactenemic birds. Therefore, a need exists to isolate, identify and regulate the structural gene encoding turkey VIP.

## 20 Summary of the Invention

The present invention provides an isolated and purified DNA molecule comprising a DNA segment encoding a turkey vasoactive intestinal peptide. The present invention also provides an isolated and purified DNA molecule consisting essentially of a DNA segment encoding (a) a turkey  
25 vasoactive intestinal peptide, (b) a turkey prepro vasoactive intestinal peptide or (c) a biologically active subunit of (a) or (b). Preferably, the present invention provides a DNA segment which consists essentially of (a) DNA of SEQ ID NO:2, (b) DNA of SEQ ID NO:7, or (c) DNA of SEQ ID NO:9.

An isolated and purified DNA molecule, such as a probe or a  
30 primer, of at least seven nucleotide bases which hybridizes to these DNA

molecules under the stringency conditions of Example 2, is also provided by the invention. The present invention also provides a probe or a primer comprising at least seven nucleotide bases of any of the above-disclosed single-stranded DNA sequences detectably labeled or having a binding site for a detectable label. As disclosed below, such probes or primers are useful to detect, quantify and amplify complementary DNA strands in avian tissue samples.

Thus, the present invention provides an isolated and purified DNA molecule comprising DNA encoding mature turkey vasoactive intestinal peptide (VIP) (SEQ ID NO:1), said VIP having the formula: His Ser Asp Ala Val Phe Thr Asp Asn Tyr Ser Arg Phe Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Asn Ser Val Leu Thr, or a biologically-active subunit thereof. Preferably, the DNA encoding the VIP of SEQ ID NO:1 consists of the nucleotide sequence of SEQ ID NO:2: CAC TCT GAT GCT GTC TTC ACT GAC AAT TAC AGC CGC TTT CGA AAG CAA ATG GCT GTG AAG AAA TAC TTA AAC TCA GTT TTA ACT, which is also depicted, along with the amino acid sequence of VIP, in Figure 1.

Representative isolated and purified DNA molecules of the invention consist of: (a) the entire DNA molecule depicted in Figure 1, (SEQ ID NO:7), as well as (b) a DNA molecule which encodes prepro VIP, or a biologically active subunit of mature VIP or prepro VIP. These DNA molecules are double-stranded or single-stranded, preferably, they are cDNA. For example, the native DNA molecule encoding turkey prepro VIP of SEQ ID NO:8 is depicted under amino acids residues 1-165, and is designated SEQ ID NO:7.

Also encompassed by the invention is an isolated and purified DNA molecule encoding mature turkey VIP which hybridizes to a DNA sequence complimentary to DNA of SEQ ID NO:5 under the high-stringency hybridization conditions disclosed herein below, or under conditions of greater stringency.

As used herein, the terms "isolated and purified" refer to *in vitro* isolation of a DNA molecule or peptide from its natural cellular environment, and

from association with other coding regions of the avian genome, so that it can be sequenced, replicated and/or expressed.

Preferably, the isolated and purified DNA sequences of the invention comprise a single coding region, and are no more than about 500-900  
5 base pairs in length. Thus, the present DNA molecules are those "consisting essentially of" or those consisting of DNA segments encoding VIP, pre-pro VIP or a biologically active subunit of either. Unexpectedly, certain DNA molecules of the invention were found to include a DNA segment encoding a peptide known as peptide histidine isoleucine (PHI), which is also within the scope of the term  
10 "turkey vasoactive intestinal peptide."

The term "biologically active" refers to a polypeptide which has at least about 50% of the *in vivo* biological activity of turkey VIP of SEQ ID NO:1, as determined by the assays disclosed in commonly-assigned U.S. patent application 07/949,797, filed September 22, 1992.

15 The present invention also provides an expression vector, preferably a linear vector, comprising an isolated DNA molecule encoding (a) turkey vasoactive intestinal peptide (VIP), said VIP having the formula of SEQ ID NO:1; (b) prepro VIP of SEQ ID NO:8; or (c) a biologically active subunit peptide of (a) or (b). Preferably, the vector comprises a single coding region, and  
20 comprises a second DNA sequence operably linked to the coding sequence, and capable of directing expression of the VIP polypeptide of (a)-(c), such as a promoter region operably linked to the 5' end of the coding DNA sequence. Such expression vectors can be constructed and employed to transform host cells, i.e., procaryotic cells, in order to produce preselected VIP peptides. Although the  
25 present vectors contain only one VIP coding region, they also can contain a DNA sequence which is a selectable marker gene or reporter gene, as described below. The expression vectors can also be constructed and employed to transform eukaryotic cells. The present invention also provides a transformed eukaryotic host cell, which host cell contains an exogenous avian vasoactive intestinal  
30 peptide gene comprising: a native vasoactive intestinal peptide gene; and



a DNA molecule encoding an exogenous avian vasoactive intestinal peptide wherein the DNA molecule expresses the exogenous avian vasoactive intestinal peptide gene at detectable levels.

The present invention also provides a method of introducing and  
5 expressing an exogenous avian vasoactive intestinal peptide gene into a host cell comprising: transforming host cells *in vitro* with an expression cassette comprising a DNA molecule encoding an avian vasoactive intestinal peptide gene operably linked to a promoter functional in the host cell; and identifying a transformed host cell which expresses the DNA molecule. This method also  
10 provides isolated recombinant avian vasoactive intestinal peptides, which are recovered as products of the transformed host cells, when the cells are cultured under appropriate conditions.

#### **Brief Description of the Figures**

15 Figure 1. Composite of the nucleotide sequence of three different overlapping tVIP cDNA clones (SEQ ID NO:7) and their corresponding inferred amino acid sequence (SEQ ID NO:8). Nucleotide residues are numbered in the 5' to 3' direction and amino acid residues are numbered beginning with the initiator methionine of the signal polypeptide. Residues presented in bold type and  
20 underlined represent the 28 amino acid tVIP. Two potential polyadenylation signals (AATAAA) are underlined. The putative PHI location, based on the PHI location in mammalian species, is marked by a double arrow.

Figure 2. Steady-state levels of hypothalamic tVIP mRNA during  
25 the reproductive cycle. Northern blots of turkey hypothalamic mRNA isolated during various portions of the reproductive cycle were first hybridized to a tVIP probe. Blots were then stripped and rehybridized with a chicken  $\beta$ -actin probe. The intensity of hybridization for tVIP and chicken  $\beta$ -actin mRNA was quantified and normalized. ADU=Arbitrary Densitometric Units. Values of 4 independent  
30 experiments with two replica are expressed as mean  $\pm$  SE. <sup>a,b,c</sup> Means with

different letters are statistically significant ( $P < 0.05$ ). A representative autoradiogram of a blot hybridized with a tVIP probe is shown below the schematic diagram. NPH=reproductively inactive. STI=photostimulated. LAY=laying. INC=incubation. REF=photorefractory.

5

Figure 3. Steady-state levels of ME VIP content (solid bar; pg/mg protein) and the associated serum PRL (cross hatched bar; ng/ml) during the reproductive cycle. Values of 4 independent experiments with two replica are expressed as mean  $\pm$  SE. <sup>a,b,c</sup> Within experiments, means with different letters are statistically significant ( $P < 0.05$ ).

10

Figure 4. Northern blot analysis of tissue-specific tVIP mRNA expression. Total cellular RNA was prepared from various turkey tissues. Twenty  $\mu$ g of total RNA was analyzed per lane with a tVIP probe.

15 HYP=hypothalamus. ON=optic nerve.

Figure 5. Schematic representation of the position of synthetic oligonucleotide primers used for RT-PCR analysis. The tVIP-specific primer pairs were selected to specifically identify tVIP mRNAs that either lacked or contained (205 bp or 306 bp product, respectively) the PHI sequence. The  $\beta$ -actin specific primer pairs were selected to generate a 581 bp product as an internal control.

20

Figure 6. RT-PCR analysis of the tissue-specific expression of alternatively spliced tVIP mRNAs. One  $\mu$ g of total RNA was reverse-transcribed into cDNA. tVIP- and  $\beta$ -actin-exon-specific primers were used to amplify tVIP- and  $\beta$ -actin-specific transcripts. Amplified DNA fragments were subjected to 2% agarose gel electrophoresis and then stained with ethidium bromide. Mobility of DNA size markers (in bp) and the expected mobilities of tVIP- and  $\beta$ -actin-specific amplified products are shown to the left and right, respectively.

30

Figure 7. Schematic representation of the relative levels of VIP mRNA in various turkey tissues as determined by RT-PCR analyses. The band intensity of amplified tVIP and  $\beta$ -actin fragments were quantified and normalized. Values of 4 independent experiments with two replica are expressed as mean  $\pm$  SE. ND=non-detectable.

Figure 8. Nucleotide sequence of the PHI-encoding exon (SEQ ID NO:9) and its corresponding inferred amino acid sequence (SEQ ID NO:10). Bolded and underlined letters represent the 27 amino acids of PHI peptide and shadowed letters represents nucleotide and amino acid sequences that flank either side of PHI.

Figure 9. RT-PCR detection of alternatively spliced preproVIP transcripts during various reproductive stages. One  $\mu$ g of total RNA was reverse-transcribed, amplified and analyzed. Mobility of DNA size markers are shown on the left. The expected mobilities of turkey VIP containing PHI or lacking PHI and  $\beta$ -actin-specific amplified products are shown to the right.

Figure 10. Schematic representation of relative tVIP mRNA levels during various reproductive stages. The band intensity of amplified tVIP which does not contain PHI sequence was quantified and normalized. Values of 4 independent experiments with two replica are expressed as mean  $\pm$  SE. <sup>a,b,c</sup> Within an experiment, means with different letters are statistically significant ( $P < 0.05$ ).

Figure 11. Schematic representation of alternatively spliced minor tVIP transcripts during various reproductive stages. The band intensity of the amplified minor tVIP containing PHI sequence was quantified and normalized (solid bar). The minor tVIP transcript values were divided by total tVIP transcript values and represented as % of total tVIP mRNA (cross-hatched bar).

Figure 12. Schematic representation of tissue specific alternative splicing pathways in the expression of the turkey preproVIP gene. In the structure of the VIP gene, boxes and lines correspond to exons and introns, respectively. Tissues in which only VIP is expressed or where both VIP and PHI are expressed are shown in parentheses.

### Detailed Description of the Invention

Specifically, the present invention provides an isolated and purified cDNA molecule such as that represented by the complete nucleotide sequence shown in Figure 1 (SEQ ID NO:7), which comprises a DNA sequence encoding prepro tVIP. The present invention further provides an isolated amino acid sequence consisting of the complete amino acid sequence shown in Figure 1 (SEQ ID NO:8), as well as to an isolated and purified DNA molecule encoding mature VIP (SEQ ID NO:1, underlined in Figure 1), such as that depicted in Figure 1. The present invention also provides an isolated and purified DNA molecule encoding turkey mature VIP or preproVIP which hybridizes to a DNA sequence complementary to the DNA sequence of SEQ ID NO:5 under stringency conditions disclosed in Example 2. Polypeptides encoded by this DNA are also within the scope of the invention which exhibit at least 50% of the biological activity of turkey mature VIP, measured as discussed below.

The present invention also provides isolated and purified DNA molecules which provide "anti-sense" mRNA transcripts of the DNA sequences shown in Fig. 1. For example, one such DNA molecule comprises a sequence consisting of the base pairs

TCAATTTTGACTCAAATTCATAAAGAAGTGTCGGTAAACGAAAGCTTTC  
GCCGACATTAACAGTCACTTCTGTCGTAGTCTCAC (SEQ ID NO:5) or a functional subunit thereof, which when expressed from an expression vector in a host avian cell, will function to block the production of avian VIP by said cell.

The polymorphic cDNA sequences of the present invention can be introduced into the genome of cell lines, whether mammalian, bacterial, or insect

cell lines, by *in vitro* techniques known in the art, to yield a transfected cell line having the cDNA stably integrated into its genome, so that the bioactive VIP molecules of the present invention are expressed by the cell lines. That is, the present invention also provides a transfected cell line having a genome augmented  
5 by a recombinant (non-native) DNA sequence, preferably by a chromosomally integrated recombinant (genetically engineered) DNA sequence that includes a gene for encoding turkey VIP, preproVIP or a bioactive subunit of either molecule.

As used herein, the term "cell line" is intended to refer to well-  
10 characterized homogenous, biologically pure populations of cells that are neoplastic or which have been "immortalized" *in vitro* by methods known in the art. The cell line is preferably of avian origin, but cell lines derived from other species may be employed, including murine, ovine, hamster, human, bovine, and the like or from prokaryotes or insects.

15 "Transfected" is used herein to include any cell or cell line, the genome of which has been altered or augmented by the presence of at least one recombinant DNA sequence, which DNA is also referred to in the art of genetic engineering as "heterologous DNA," "exogenous DNA," "genetically engineered," "non-native," or "foreign DNA," wherein said DNA was isolated and introduced  
20 into the genome of the cell or cell line by a process of genetic engineering. The cell lines of the present invention are typically produced by transfection with a DNA sequence in a plasmid expression vector, a viral expression vector, or as an isolated linear DNA sequence.

As used herein, the term "recombinant DNA" refers to DNA that  
25 has been derived or isolated from any appropriate tissue source, that may be subsequently chemically altered *in vitro*, and later introduced into host cells. An example of recombinant DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. Another example  
30 of recombinant DNA "derived" from a source, would be a cDNA sequence that is

prepared from isolated RNA. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering. Therefore, "recombinant DNA" includes completely synthetic DNA sequences, semi-synthetic DNA sequences, chimeric DNA sequences, DNA sequences isolated and purified from biological sources, and DNA sequences derived *in vitro* from RNA, as well as mixtures thereof. Generally, the recombinant DNA sequence is not originally resident in the genome which is the recipient of the DNA, or it is resident in the genome but is not expressed. As used herein, the term "chimeric" DNA sequence or molecule, refers to a DNA molecule comprising sequences derived from the genomes of two or more species that do not exchange DNA under normal conditions, or to DNA sequences which are linked in a manner that does not normally occur in the native genome.

The recombinant DNA sequence, used for transfection herein, may be circular or linear, double-stranded or single-stranded. Generally, the DNA sequence is in the form of chimeric DNA, such as plasmid DNA, that can also contain coding regions flanked by regulatory sequences which promote the expression of the recombinant DNA present in the progeny of the transfected host cell. For example, the recombinant DNA may itself comprise a promoter that is active in the host cells, or may utilize a promoter already present in the genome that is the transfection target.

The general methods for constructing recombinant DNA which can transfect target cells are well known to those skilled in the art, and the same conditions and methods of construction may be utilized to produce the DNA useful herein. For example, J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (2nd ed., 1989), provides suitable methods of construction.

Moreover, the general methods for isolating and purifying a recombinantly expressed protein from a host cell are well known to those in the art. Examples of the isolation and purification of such proteins are given in Sambrook et al.

5           Aside from recombinant DNA sequences that serve as transcription units for VIP or other portions thereof, a portion of the recombinant DNA may be untranscribed, serving a regulatory or a structural function. The recombinant DNA to be introduced into the cells further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and  
10 selection of transfectants. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in cells, particularly avian or mammalian cells. Useful selectable markers are well known in the art and include, for example, antibiotic  
15 and herbicide resistance genes. Preferred selectable markers for use in the transfection processes of the present invention include resistance to the antibiotic neomycin or hygromycin, or to herbicides such as glyphosate, phosphinothricin, and the like.

Reporter genes are used for identifying potentially transformed  
20 cells and for evaluating the functionality of regulatory sequences. Reporter genes that encode for easily assayable marker proteins are well known in the art. In general, a reporter gene is a gene that is not present in, or expressed by, the recipient organism or tissue and that encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Preferred  
25 reporter genes for use in the transformation processes of the present invention include  $\beta$ -galactosidase, luciferase or chloramphenicol acetyltransferase.

Other elements such as introns, enhancers, polyadenylation sequences and the like, can also be part of the recombinant DNA sequence. Such elements may or may not be necessary for the function of the DNA, but may  
30 provide improved expression of the DNA by affecting transcription. stability of

the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the cell.

Sources of nucleotide sequences useful in the present invention include PolyA<sup>+</sup> RNA from avian cells, from which the mRNA encoding the VIP  
5 can be derived and used for the synthesis of the corresponding cDNA by methods known in the art. Such sources include turkey hypothalami, for example.

The recombinant DNA can be readily introduced into the target cells by transfection with the expression vector comprising cDNA encoding the turkey VIP by the calcium phosphate procedure of C. Chen et al., Mol. Cell  
10 Biol., 7, 2745 (1987). Transfection can also be accomplished by lipofection, using commercially available kits, e.g., provided by BRL, or electroporation.

The invention will be further described by reference to the following detailed examples.

#### 15 Example 1. VIP cDNA Isolation and Sequence Analysis

Nicholas Large White female turkeys were used throughout these studies. All birds were reared and housed in floor pens with trap nests. Feed and water were constantly available.

Total RNA was isolated from the hypothalami of incubating turkey  
20 hens using the cesium chloride-guanidinium isothiocyanate method (see, J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989) at 7.19 - 7.29). Poly(A)<sup>+</sup> RNA was isolated by chromatography on oligo (dT) cellulose. A custom cDNA library was constructed by InVitrogen (San Diego, California) in the phagemid vector  
25 pcDNAII. Positive colonies were isolated by colony screening on Colony/Plague Screen (Dupont, Boston, Massachusetts) using a murine VIP cDNA as described in Lamperti et al. (Mol. Brain Res., 9, 217 (1991)). Only about 150 bp of the VIP coding region was labeled with [ $\alpha$ -<sup>32</sup>P] dATP, [ $\alpha$ -<sup>32</sup>P] dCTP, [ $\alpha$ -<sup>32</sup>P] dGTP and [ $\alpha$ -<sup>32</sup>P] TTP (Amersham Life Science, Arlington Heights, Illinois) using VIP-  
30 specific primers in a polymerase chain reaction (PCR).



The three largest putative clones (tVIP-1, -4, and -5) were selected for nucleotide sequence analysis. Both strands of each clone were sequenced using the dideoxy chain termination method with both the modified T7 polymerase (Sequenase; U.S. Biochemical, Cleveland, OH) and Taq DNA polymerase. Autoradiographs of sequencing gels were read manually or on a Applied Biosystems Model 373A (Microchemical Facility, University of Minnesota). The nucleotide and predicted amino acid sequences were compiled, analyzed, and compared with mammalian VIP cDNA sequences by using the homology search feature of the Intelligenetics Program.

Figure 1 shows the complete nucleotide sequence of a composite sequence of the three different tVIP cDNA clones (SEQ. ID NO: 7). tVIP-1 and tVIP-5 were partial sequences beginning within the coding region at amino acid 30, whereas tVIP-4 included all of the 5' coding region plus an additional portion of 5' untranslated region (UTR). Otherwise, these three different clones were found to be identical when overlapping sequences were compared. The tVIP-4 clone contained 27 base pairs (bp) of the 5'-UTR, 495 bp of coding sequence, 282 bp of 3'-UTR and a 8 bp polyA track, while clones tVIP-1 and tVIP-5 lacked the 5' portion of the sequence.

The amino acid sequence (residues 94-121, bolded and underlined) deduced from nucleotide residues 307-390 showed high similarity to rat and human VIP (Nishizama et al., FEBS Lett., 183, 55 (1985); Itoh et al., Nature, 304, 547 (1983)). As shown in Figure 1, the 27 amino acid residue sequence of PHI-27 as well as the 8 additional amino acids flanking either side of PHI-27 in rat and human (Nishizama et al., FEBS Lett., 183, 55 (1985); Itoh et al., Nature, 304, 547 (1983)) were not present in tVIP clones. Glycine together with the basic amino acids Lys and Arg form a carboxy-terminal amidation signal sequence (Bradbury et al., Nature, 298, 686 (1982)), which lies adjacent to the carboxy-terminus of VIP. The 3'-UTR of tVIP clones had two potential polyadenylation sites, AATAAA (Figure 1, bolded and underlined, nucleotide residues 662-667 and 774-779). Northern blot analysis (Figures 2 and 4) revealed

a processed tVIP transcript of approximately 900 bases which hybridized to the <sup>32</sup>P-labeled tVIP cDNA probe.

**Example 2. Assay for Steady State Hypothalamic tVIP mRNA Abundance**

5                   Steady-state levels of hypothalamic tVIP mRNA were measured by Northern blot analysis using total cellular RNA isolated from turkeys at different reproductive stages (Figure 2). The reproductive groups used were 1) reproductively inactive (nonphotostimulated) hens maintained under a 6L:18D lighting regime for at least 8 weeks, 2) photostimulated hens that had been  
10 switched from 6L:18D to 16L:8D lighting schedule for 10 days, 3) laying hens that were in the nest only once or twice a day and laid regularly, 4) incubating birds that were in the nest six times per day for at least two weeks and did not lay during that time but were allowed to remain in the nest, 5) incubating birds as in 4) but were nest deprived by transferring the hens to individual wire cages, 6)  
15 photorefractory hens that were maintained on a 16L:8D lighting schedule that had ceased nesting and egg laying and had completed molting. Blood samples were collected at various times. Upon sacrifice, the median eminence and hypothalamus were frozen in liquid nitrogen.

                  Total RNA was extracted from frozen tissues using guanidinium  
20 thiocyanate-phenol-chloroform method of P. Chomczynski et al. (Anal. Biochem., 162, 156 (1987)). Total RNA was prepared for electrophoresis by the method of R.M. Forney et al. (BRL Focus, 10, 5 (1988)). Ten micrograms of total RNA was loaded per lane and fractionated on 1% agarose gels containing 2.2 M formaldehyde. RNA was transferred to Gene Screen membranes (Dupont,  
25 Boston, MA) by capillary action. The filter was prehybridized for 6 to 8 hours at 42°C in a solution containing 50% formamide, 5xSSC (1xSSC=0.15M NaCl, 0.015M Na Citrate, pH 7.0), 1% SDS, 2x Denhardt's solution. and 25 µg/ml tRNA.

                  Blots were hybridized with the turkey VIP cDNA of Figure 1  
30 labeled by nick translation (with [α-<sup>32</sup>P] dCTP (ICN, Irvine, CA)). according to

the methodology of Sanger et al. (Proc. Nat'l Acad. Sci., 74, 5463 (1977)).

Hybridization and washing was conducted according to the manufacturer's directions under high stringency conditions. High stringency conditions are defined as hybridization of membranes at 42°C for 16-18 hours in 50%

- 5 formamide, 5xSSC, 1% SDS, 2x Denhardt's solution, 25 µg/ml tRNA, and washing once for 5 minutes in 2xSSC at room temperature (25°C), once for 45 minutes in 2xSSC, 1% SDS at 52°C and once for 45 minutes in 0.2xSSC, 1% SDS at 52°C. Membranes were air-dried and exposed to Kodak XAR-5 film with intensifying screens at -80°C. After autoradiography, the membranes were boiled  
10 for 30 min in 0.1xSSC plus 1% SDS or in 0.02xSSC plus 0.1% SDS at 70°C for 1 hour to remove the VIP probe and then rehybridized with a nick translated chicken β-actin probe, using the methodology of Kost et al. (NAR, 11, 8287 (1983)). The preparation of these reagents is disclosed in Cloning Manual, cited hereinabove, or in the 1982 edition. Some of the reagents are available from  
15 Sigma Chemical Company (St. Louis, Missouri).

- The band intensity of hybridizing VIP mRNA and β-actin transcripts was quantified from autoradiographs using a scanning densitometer (Model 4000, Ambis, Inc., San Diego, California), and normalized to the band intensity of the β-actin mRNA band. Values were expressed as arbitrary  
20 densitometric units (ADU). The results are shown in Figure 2.

- Hypothalamic tVIP mRNA abundance was lowest in the reproductively inactive (NPH) and photostimulated (STI) hens but increased in laying (LAY) hens who exhibited 2.1- and 2.4-fold greater steady-state tVIP mRNA levels than the NPH and STI birds, respectively. The highest level of  
25 hypothalamic tVIP mRNA content was observed during the incubation (INC) stage (3.0-, 3.4- and 1.4-fold greater than NPH, STI and LAY, respectively). No significant difference in the steady-state tVIP mRNA levels, however, was observed between INC and photorefractory (REF) birds.

### Example 3. Median Eminence VIP Content and Serum Prolactin Levels

The ME content and serum prolactin levels during various stages of the reproductive cycle were analyzed. Serum prolactin concentrations were measured using a homologous radioimmunoassay (RIA) employing the methodology of J.A. Proudman et al. (Biol. Reprod., 25, 573 (1981)). VIP was extracted from the median eminence with acetic acid and measured using a homologous radioimmunoassay, as disclosed by L. J. Mauro et al. (Gen. Comp. Endoc., 87, 481 (1992)). The results are shown in Figure 3, wherein the left bar of each pair of bars represents the PRL level (ng/ml) and the right bar represents the VIP level (ng/ml).

The ME VIP content (Figure 3) increased gradually during the reproductive cycle, similar to that shown in the steady-state tVIP mRNA levels (Figure 2) during reproductive cycles. VIP concentrations in the ME were lowest in the NPH and increased 1.4-fold in STI hens. An additional increase was seen in LAY hens who exhibited VIP concentrations 2.2- and 1.8-fold greater than NPH and STI birds, respectively. Highest VIP content in the ME was observed in INC hens (4.2-, 2.9- and 1.8-fold greater than NPH, STI, and LAY, respectively).

In some instances, the changes in hypothalamic VIP contents and levels of tVIP expression were correlated with elevated circulating PRL levels (Figure 3). Increased ME VIP contents in STI hens (1.4-fold increase) were associated with higher serum PRL levels (2.1-fold increase) compared to those of NPH. Further increases in VIP contents in the ME (2.3- and 1.7-fold) and steady-state hypothalamic tVIP mRNA levels (2.1- and 2.4-fold) in LAY hens were associated with a higher serum PRL levels (24- and 12-fold increases) compared to those of NPH and STI hens, respectively. These increases in VIP expression were even higher in INC hens which correlated well with increased serum PRL levels (3.0-, 3.4-, and 1.4-fold increases in the steady-state hypothalamic tVIP mRNA levels; 4.2-, 2.9-, and 1.8-fold increases in the ME VIP contents; and 219-, 105-, 9-fold increases in the serum PRL levels compared

to those of NPH, STI and LAY, respectively). While steady-state hypothalamic tVIP mRNA level remained constant and ME VIP content was slightly decreased (1.8-fold), a precipitous decrease in serum PRL level (42-fold) was seen in REF birds when compared to INC.

5           The data indicates that hypothalamic VIP concentrations and VIP mRNA contents were highest during the incubation phase of the reproductive cycle. Highest levels of serum PRL were also observed during this period.

**Example 4. Tissue Specific Levels of Turkey VIP mRNA**  
**Detected by RT-PCR**

10           Initially, tissue-specific tVIP mRNA expression was analyzed by Northern blot analysis using total cellular RNA isolated from various turkey tissues. Only the hypothalamus and the optic nerve (Figure 4) showed tVIP mRNA expression even though all tissues examined expressed  $\beta$ -actin mRNA.

15           Mammalian preproVIP (Nishizama et al., FEBS Lett., **183**, 55 (1985); Itoh et al., Nature, **304**, 547 (1983)) transcripts have been shown to encode both peptide histidine isoleucine (PHI) and VIP peptides whereas the tVIP clones were found to contain only the VIP peptide (Figure 1). To identify tissue-specific splicing patterns which might include the PHI sequence, a series of PCR  
20 analyses from reverse-transcribed RNA (Figures 5, 6 and 7) were performed.

          One  $\mu$ g of total cellular RNA from various tissues was reverse transcribed by Moloney murine leukemia virus reverse-transcriptase using oligo (dT) as a primer as recommended by Perkin Elmer Cetus (Norwalk, Connecticut). Reaction mixtures (50  $\mu$ l) were prepared using the Ampliwax hot start technique  
25 as recommended by the manufacturer (Perkin Elmer Cetus). The VIP- and  $\beta$ -actin-specific oligonucleotide primers used in the coamplification PCR reaction are: 1) VIP, a) 5'-TGAGGTAAAGTATTTCTTCACAGCCATTTGCTT (SEQ. ID NO: 11); b) 5'-GACCGCGCCCATGGGTCCCTAAAGTC (SEQ. ID NO: 12);  
2)  $\beta$ -actin, a) 5'-ACCAGTAATTGGTACCGGCTCCTC (SEQ. ID NO: 13); b) 5'-  
30 TCTGGTGGTACCACAATGTACCCT (SEQ. ID NO: 14). The tVIP-specific

primer pairs (SEQ. ID NO: 11 and 12) were selected to specifically identify tVIP mRNAs that were alternatively spliced and either lacked or contained the PHI sequence (either a 205 bp or 306 bp product, respectively). As an internal control for RT-PCR,  $\beta$ -actin-specific primer pairs (SEQ. ID NO:13 and 14) were employed (Figure 5).

The amplification profile consisted of 30 cycles of 1 minute at 95°C, 1 minute at 65°C, and 1 minute at 72°C. The products of the reaction were analyzed by electrophoresis through 2% agarose and visualized by staining with ethidium bromide. After Southern blot analysis to verify VIP- and  $\beta$ -actin-specific amplification, the steady-state levels of VIP and  $\beta$ -actin mRNA by RT-PCR were quantified densitometrically by the band intensity of VIP and  $\beta$ -actin RT-PCR fragments and normalized to the band intensity of the  $\beta$ -actin.

The tVIP mRNA expression was highest in the hypothalamus ( $1.8 \pm 0.18/\text{ADU}$ ) followed by small intestine ( $1.1 \pm 0.23/\text{ADU}$ ) and the optic nerve ( $1.0 \pm 0.26/\text{ADU}$ ). Detectable levels were also observed in the cortex (COR), anterior pituitary (A.P.), colon (COL) and uterus (UTE). The cerebrum (CER), shell gland (S.G.) and oviduct (OVI) showed lower levels of tVIP mRNA expression (Figures 6 and 7).

Tissue-specific differential expression of transcripts that coded for either VIP or both VIP and PHI peptides were observed. One transcript that was observed in every tissue showed VIP expression but lacked PHI, while the other larger transcript (observed only in the hypothalamus) encoded both VIP and PHI peptides (Figure 6). However, PHI-specific primer pairs did not produce a transcript that specifically encoded PHI but lacked VIP in any of the tissues examined in this study.

While alternatively spliced transcripts could not be detected by Northern blot analysis due to the small differences in the size of the transcripts, RT-PCR analysis was able to quantify alternatively spliced mRNAs. The PCR amplification rate of the two different products was tested for different cycling times and these values were analyzed, and normalized. The slope of the

amplification rate curves of these two different products remained constant through 24 to 35 cycles with a similar amplification efficiency for the two tVIP transcripts and  $\beta$ -actin-specific fragments (no significant changes were observed among those values obtained from different cycling parameters). Therefore, the initial relative amounts of the different mRNAs in the sample should be identical to the numerical values as determined for the corresponding PCR products.

The approximate 300 bp PCR fragment was isolated, purified and subsequently subcloned, followed by nucleotide sequence analysis. This fragment was identified as the alternatively spliced PHI containing sequence which encoded the 27 amino acid PHI peptide, as well as 4 amino acids that flanked both sides of PHI (Figure 8). PCR amplification of turkey genomic DNA and partial sequence analysis showed the PHI encoding exon (105 bp) was flanked by introns on either side, with the VIP encoding exon located down stream of the PHI encoding exon. Thus, the turkey preproVIP gene encodes both VIP and PHI which are separated by at least one intron.

#### **Example 5. Quantification of Alternatively Spliced PreproVIP Transcripts during Various Reproductive Stages**

To determine if the alternatively spliced transcripts were equally expressed during various reproductive stages, levels of the major hypothalamic tVIP transcript were analyzed by RT-PCR (Figures 9 and 10). Low steady-state levels of the major hypothalamic tVIP mRNA were observed in NPH ( $4.4 \pm 0.9/\text{ADU}$ ) and STI ( $5.1 \pm 0.8/\text{ADU}$ ). A significant increase (1.7- and 1.5-fold) was seen in LAY hens ( $7.6 \pm 0.7/\text{ADU}$ ) compared to the NPH and STI birds, respectively. Highest steady-state levels of major hypothalamic tVIP mRNA was observed in INC birds. However, no significant difference was seen between INC and REF hens ( $10.5 \pm 1.4/\text{ADU}$  and  $9.5 \pm 1.1/\text{ADU}$ , respectively). Nest deprivation of INC, a procedure known to reduce circulating PRL, significantly decreased steady-state hypothalamic major tVIP mRNA content (NDP;  $3.9 \pm 2.1/\text{ADU}$ , 2.7-fold decrease compared to INC hens).

Expressed levels of the minor hypothalamic tVIP mRNA transcript containing both PHI- and VIP-encoding exons were also analyzed by RT-PCR as described above (Figure 11). In contrast to the gradual increase observed for the steady-state levels of the major hypothalamic tVIP mRNA during reproductive stages, neither the steady-state levels or relative amount of this transcript (as represented as percent of total tVIP mRNA) showed significant changes between reproductive stages.

### DISCUSSION

The nucleotide sequence of the tVIP cDNA shares some nucleotide similarity to rat and human VIP cDNAs (Nishizama et al., FEBS Lett., 183, 55 (1985); Itoh et al., Nature, 304, 547 (1983)). Nucleotides 25 through 31 of prepro tVIP (GCCATG; Figure 1) conformed closely to the translation initiation consensus sequences (ACCATGG) described by Kozak (Cell, 44, 283 (1986)). Two potential polyadenylation signal sequences AATAAA were located 143 and 20 bases upstream from the poly A track.

Comparison of tVIP precursor molecule with the rat and human VIP precursor revealed that the processing sites for generation of VIP and PHI (or PHM) showed a high degree of similarity. This suggests that both turkey VIP and PHI are generated from their precursor by proteolytic processing, and that the peptides are carboxy-terminally amidated. The amino acid sequence similarity of tVIP to rat and human VIP is 69%. The 27 amino acids of turkey PHI, however, showed less sequence similarity (60%) compared to rat and human PHI.

Quantification of the steady-state hypothalamic tVIP mRNA and VIP content in the ME during a photo-induced reproductive cycle revealed a gradual increase in VIP gene expression, plateauing with highest levels in the INC and REF birds. The changes in VIP gene expression were associated with a coincident increase in circulating PRL. Thus, under conditions of hyperprolactinemia, hypothalamic VIP transcription is high. These results together with the finding that PRL transcription and pituitary PRL levels are



greatest in lactotrophs from hyperprolactinemic incubating hens (Wong et al., Biol. Reprod., 47, 598 (1992)) indicate that changes have occurred within the central VIPergic system as well as the pituitary of incubating hens to enhance PRL secretion. Furthermore, these findings suggest interactive regulatory  
5 mechanism(s) between both VIP and PRL gene expression. In this context, VIP elevates PRL mRNA levels *in vivo* (Talbot et al., Endocrinol., 129, 496 (1991); Pitts et al., Biol. Reprod., 50, 1344 (1994); Pitts et al., Biol. Reprod., 50, 1350 (1994)) and *in vitro* (Xu et al., Poult. Sci., 71(5), 62 (1992)). Finally, it has been demonstrated that immunoneutralization of VIP reduced pituitary PRL  
10 content (Sharp et al., J. Endocrinol., 122, 5 (1989)) and mRNA levels (Talbot et al., Endocrinol., 129, 496 (1991)).

The incubation behavior-associated increase in PRL expression is maintained at high levels as long as nesting activity persists. Depriving incubating birds of access to nests lowers PRL levels (Mauro et al., Endocrinol.,  
15 125, 1795 (1989); Talbot et al., Endocrinol., 129, 496 (1991)) and hypothalamic VIP-immunoreactivity (Mauro et al., Endocrinol., 125, 1795 (1989)). These findings are consistent with the decline in hypothalamic VIP gene expression after nest-deprivation observed in the present study and indicates the importance of the nesting stimulus in maintaining elevated circulating PRL and hypothalamic VIP  
20 mRNA. The mechanism by which nesting activity modulates VIPergic gene expression remains an open question.

The elevated ME VIP content and steady-state tVIP mRNA levels combined with low circulating PRL levels were observed in the REF birds. This apparent discrepancy between VIP expression and PRL secretion could result  
25 from a decrease in pituitary responsiveness, since pituitary cells from REF birds exhibited the lowest VIP-induced PRL release (El Halawani et al., Gen. Comp. Endocrinol., 80, 138 (1990)) and lowest pituitary VIP receptors (Rozenboim et al., Biol. Reprod., 18, 1129 (1993)) when compared to laying and incubating birds. The non-photostimulated birds exhibited both low ME VIP content and  
30 steady-state tVIP mRNA levels indicating that there is a decline in VIP

expression when photorefractory birds are transferred from a gonadal stimulatory lighting schedule of 16L:8D to a short day light regimen. The modulation of VIP gene expression by photic information is supported by the increase in hypothalamic VIP immunoreactivity following photostimulation (Mauro et al., *Endocrinol.*, 125, 1795 (1989); L.J. Mauro et al., *Gen. Comp. Endoc.*, 87, 481 (1992)). Further evidence is provided by the presence of opsin-like pigment in VIP neurons within the infundibular nuclear complex, a region thought to be the site of extraretinal hypothalamic photo-receptor (Silver et al., *Cell Tissue Res.*, 253, 189 (1988)). In the mammalian circadian oscillator, the suprachiasmatic nucleus (SCN), VIP positive neurons receive direct neural inputs from retinal ganglion cells (Ibata et al., *Neuro. Sci. Lett.*, 97, 1 (1989)). Manipulation of photic information, by optic tract transection or change in environmental lighting, can modify VIP gene expression within these ventrolateral SCN neurons (Albers et al., *Brain Research*, 437, 189 (1987); Stopa et al., *Mol. Brain Res.*, 4, 319 (1990)). The finding of this study lends support to a hypothetical scheme for VIP gene expression that is intimately linked to photoperiodic mechanisms active during reproduction. Such a model would seem appropriate in view of the fact that VIP is the most important PRF in birds and PRL is a hormone whose secretion is seasonally or photoperiodically dependent in many avian species (Nicholls et al., *Physiol. Rev.*, 68, 133 (1985)).

Although Mauro et al. (*Gen. Comp. Endoc.*, 87, 481 (1992)) demonstrated VIP immunoreactivity in several tissues, the data presented herein showed that only two tissues (optic nerve and hypothalamus) expressed VIP. In addition, the fact that none of the three different tVIP cDNA clones isolated contained the PHI-encoding exon suggests that the tVIP transcript could encode only one of the turkey neuropeptides, VIP. A highly sensitive and quantitative RT-PCR assay was developed to assess if potential alternative splicing patterns of tVIP transcripts occurred in a tissue-specific manner and to determine the variation of hypothalamic tVIP mRNA levels during various reproductive stages in the domestic female turkey. As was shown for a similar RT-PCR assay for

detection of pit-1 mRNA isoforms (Day et al., Mol. Endocrinol., 8, 374 (1994)), the relative amounts of individual mRNAs obtained by use of such a method are highly accurate and reproducible (Sandbrook et al., J. Biol. Chem., 269, 1510 (1994); Goetzl et al., J. Biol. Chem., 263 9083 (1988)).

5           The RT-PCR-amplified product of total RNA showed one band of the proper size for VIP lacking the PHI-encoding exon that was present in all tissues. A second amplified product found only in hypothalamic tissue was the correct size for VIP if it contained the PHI-encoding exon. The fragment was cloned and subjected to sequence analysis and found to correspond to the  
10       expected PHI portion of the preproVIP molecule.

          The sequence encoding VIP and PHI are contained in two separate exons and the gene coding for VIP also codes for a VIP-related peptide PHI-27 (peptide-histidine-isoleucine amide) in rat or PHM-27 (peptide-histidine-methionine amide) in human. Such a tissue-specific alternative splicing  
15       mechanism of the preproVIP mRNA could give rise to polypeptide precursors containing only PHI or VIP, or both.

          The arrangement of splice sites for the PHI and VIP exons would allow either exon sequence to be removed from a transcript without altering the reading frame of the remaining message. These exons would thus act as separate  
20       functional units, each encoding a separate neuropeptide complete with the peptide cleavage signals necessary to remove it from the precursor; one unit would be selectively removed from transcripts in a given tissue to cause tissue-specific expression of the other neuropeptide.

          Using oligonucleotide and complimentary RNA probes, other  
25       investigators (Gozes et al., Peptides, 7(SI), 1 (1986); Gozes et al., Ann. NY Acad. Sci., 527, 77 (1988); Goetzl et al., J. Biol. Chem., 263, 9083 (1985); Gozes et al., J. Cell. Biochem., 26, 21 (1984)) have described multiple VIP messages in mammalian tissues, which were considered to be either partially spliced or intact initial transcripts. One mRNA (from a human tumor) hybridized selectively to  
30       VIP-specific but not to PHI-specific oligonucleotides. However, a tissue-specific

alternative splicing mechanism to produce a transcript encoding only one of the neuropeptides (PHI or VIP) was not found in the mammalian preproVIP gene. In mammals, there is no conclusive evidence for differential splicing to produce messages selectively expressing either PHI or VIP but rather the mature  
5 preproVIP mRNA contains both VIP and PHI coding sequences.

PHI and VIP have similar actions on a variety of potential target tissues (Tatemoto, Peptides, 5, 151 (1984)). PHI has been shown to be as potent as VIP in stimulating the release of PRL from dispersed rat anterior pituitary cells as well as from hemipituitaries (Werner et al., Neuroendo., 37, 476 (1983);  
10 Samson et al., Peptides, 4, 817 (1983); Kaji et al., LifeSci., 35, 641 (1984); Ohta et al., Peptides, 6, 709 (1989)). Although immunoreactive PHI and VIP are found in the same tissues, the relative levels of the two neuropeptides seem to vary substantially among different tissues (Christofides et al., Peptides, 5, 261 (1984)).

15 Radioimmunoassays have indicated that PHI and VIP concentrations are not equimolar in many mammalian tissues; VIP is significantly more abundant in certain parts of the gut, notably the stomach, and in the nasal mucosa and urogenital system (Christofides et al., Peptides, 5, 261 (1984)). These quantitative differences appear to be due to tissue-specific variations in  
20 post-translational processing (Yiangou et al., Gastroenterol., 89, 516 (1985); Yiangou et al., BBRC, 139, 1142 (1986); Yiangou et al., J. Biol. Chem., 262, 14010 (1987); Fahrenkrig et al., Regul. Peptides, 12, 21 (1985)). Such regional variations in post-translational processing have been demonstrated for proopiomelanocortin in the pituitary and for proenkephalin in the hypothalamus  
25 and adrenal medulla (Douglas et al., Ann. Rev. Biochem., 53, 665 (1984)). However, the differential splicing of tVIP transcripts in a tissue-specific fashion demonstrated in this study may provide an additional explanation for the quantitative differences in tissue concentrations of PHI and VIP.

The levels of alternatively spliced tVIP transcripts containing both  
30 the PHI- and VIP-encoding exons did not significantly change between

reproductive stages. These levels were maintained at approximately 4-6% of the total tVIP transcripts. These results strongly support the view that VIP is the predominant PRF in the adult turkey. Even though the minor PHI-VIP transcript exists at very low steady-state levels ( and PHI peptide and PRL levels appears to be uncorrelated), the possibility still exists that the minor tVIP transcript might have physiological relevance if it were selectively induced by some factor or if its stability or translational efficiency differed from the major tVIP transcript which lacks the PHI-encoding exon. Also, it will be of interest to clarify its role, if any, in PRL regulation.

Based on the sequence similarity between the avian and mammalian PHI sequence, the closing of the tVIP cDNA permits the study of the action and physiological role of PHI in birds. The role of PHI can be complemented by VIP during developmental stages and replaced by VIP in a tissue-specific manner. Revealing the tissue-distribution of VIP gene expression and tissue-specific alternative splicing contributes to an understanding of the physiological functions of the products of two alternatively spliced tVIP mRNAs as well as their relative roles in PRL regulation.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

## SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT/REGENTS OF THE UNIVERSITY OF MINNESOTA  
MOHAMED E. EL HALAWANI
- (ii) TITLE OF INVENTION: DNA ENCODING TURKEY HYPOTHALAMIC VASOACTIVE  
INTESTINAL PEPTIDE
- (iii) NUMBER OF SEQUENCES: 11
- (iv) CORRESPONDENCE ADDRESS:
  - (A) NAME: SCHWEGMAN LUNDBERG & WOESSNER, P.A.
  - (B) STREET: 3500 IDS CENTER
  - (C) CITY: MINNEAPOLIS
  - (D) STATE: MINNESOTA
  - (E) COUNTRY: USA
  - (F) POSTAL CODE (ZIP): 55402
  - (G) TELEPHONE: 612-339-0331
  - (H) TELEFAX: 612-339-3061
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Daniel J. Kluth
  - (B) REGISTRATION NUMBER: 32,146
  - (C) REFERENCE/DOCKET NUMBER: 600.321WO1

(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 28 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

His Ser Asp Ala Val Phe Thr Asp Asn Tyr Ser Arg Phe Arg Lys Gln  
1 5 10 15

Met Ala Val Lys Lys Tyr Leu Asn Ser Val Leu Thr  
20 25

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 84 base pairs

27

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CACTCTGATG CTGTCTTCAC TGACAATTAC AGCCGCTTTC GAAAGCAAAT GGCTGTGAAG 60  
AAATACTTAA ACTCAGTTTT AACT 84

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 84 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCAATTTTGA CTCAAATTCA TAAAGAAGTG TCGGTAAACG AAAGTTTTCG CCGACATTAA 60  
CAGTCACTTC TGTCGTAGTC TCAC 84

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 165 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Glu His Arg Gly Thr Ser Pro Leu Leu Leu Ala Leu Ala Leu Leu  
1 5 10 15  
Ser Ala Leu Cys Trp Arg Ala Arg Ala Leu Pro Pro Arg Gly Ala Ala  
20 25 30  
Phe Pro Ala Val Pro Arg Leu Gly Asn Arg Leu Pro Phe Asp Ala Ala  
35 40 45  
Ser Glu Ser Asp Arg Ala His Gly Ser Leu Lys Ser Glu Ser Asp Ile  
50 55 60  
Leu Gln Asn Thr Leu Pro Glu Asn Glu Lys Phe Tyr Phe Asp Leu Ser  
65 70 75 80  
Arg Ile Ile Asp Ser Ser Gln Asp Ser Pro Val Lys Arg His Ser Asp  
85 90 95

28

Ala Val Phe Thr Asp Asn Tyr Ser Arg Phe Arg Lys Gln Met Ala Val  
 100 105 110  
 Lys Lys Tyr Leu Asn Ser Val Leu Thr Gly Lys Arg Ser Gln Glu Glu  
 115 120 125  
 Leu Asn Pro Ala Lys Leu Arg Asp Glu Ala Glu Ile Leu Glu Pro Ser  
 130 135 140  
 Phe Ser Glu Asn Tyr Asp Asp Val Ser Val Asp Glu Leu Leu Ser His  
 145 150 155 160  
 Leu Pro Leu Asp Leu  
 165

## (2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 812 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGACGGATTG GCGGCTCCGT GGCGGCCATG GAGCACGGCG GCACCTCCCC GCTCCTCCTC 60  
 GCCCTGCCCC TCCTCAGCGC CCTCTGCTGG AGGGCGGGG CGCTGCCCCC GCGGGGCGCC 120  
 GCCTTCCCTG CTGTGCGCG ACTGGGAAAC AGACTGCCCT TTGATGCAGC CAGTGAATCT 180  
 GACCGGCCCC ATGGGTCCCT AAAGTCTGAA TCAGACATTT TGCAGAACAC ACTACCTGAA 240  
 AATGAGAAAT TCTATTTTGA TTTGTCCAGA ATTATGATA GCTCCCAGGA CAGTCTGTTC 300  
 AAACGCCACT CTGATGCTGT CTTCACTGAC AATTACAGCC GCTTTCGAAA GCAAATGGCT 360  
 GTGAAGAAAT ACTTAAACTC AGTTTTAACT GGAAAACGAA GCCAGGAAGA GTTAAATCCA 420  
 GCCAACTTG CAGATGAAGC AGAAATTCTT GAACCTTCCT TTTCAGAAAA CTATGATGAT 480  
 GTTCTGTAG ATGAACTGCT GAGCCACCTC CCATTGGACC TCTGAAGGAC ACCTAGAAAA 540  
 CTCTTCAACA AGAACAAGTT ATTTTTGAGT TCCACATAGT ATTTCAAAGA GATGACTTTA 600  
 GTCATCAAAT CTGAACAAAT ATGTTGTGAA GTGAAAGTTG TGATATATTT ATTTCTTATG 660  
 TAATAAAAGT TGATATTTAC ATTGTAAATA CTGTTCTAGA GTTCTCTACT GAAAGCTGTA 720  
 CATATGGATG CCAGTTAAAC AAATGAGAAG TCTGTAAGTC CATATGCTGT AAATCCTTTA 780  
 CTTCAATAAA TTCATTTGAA AATGAAAAAA AA 812

## (2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 105 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)



## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGAAATGCAA GGCATGCTGA TGGAATTTTC ACCACTGTAT ACAGCCATCT TTTGGCTAAA 60  
 CTCGCTGTGA AGAGATATCT GCATTGCTT ATTAGAAAAA GAGTT 105

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Arg Asn Ala Arg His Ala Asp Gly Ile Phe Thr Trp Val Tyr Ser His  
 1                      5                      10                      15  
 Leu Leu Ala Lys Leu Ala Val Lys Arg Tyr Leu His Ser Leu Ile Arg  
                     20                      25                      30  
 Lys Arg Val  
                     35

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGAGGTTAAG TATTCTTCA CAGCCATTG CTT 33

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GACCGCGCCC ATGGGTCCCT AAAGTC 26

30

## (2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACCAGTAATT GGTACCGGCT CCTC

24

## (2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCTGGTGGTA CCACAATGTA CCCT

24

**WHAT IS CLAIMED IS:**

1. An isolated and purified DNA molecule comprising a DNA segment encoding a turkey vasoactive intestinal peptide.
2. An isolated and purified DNA molecule consisting essentially of a DNA segment encoding (a) a turkey vasoactive intestinal peptide, (b) a turkey prepro vasoactive intestinal peptide or (c) a biologically active subunit of (a) or (b).
3. An isolated and purified DNA molecule consisting of a DNA sequence encoding a turkey vasoactive peptide of SEQ. ID NO: 1.
4. The DNA segment of claims 1, 2 or 3 which consists essentially of (a) DNA of SEQ ID NO:2, (b) DNA of SEQ ID NO:7, or (c) DNA of SEQ ID NO:9.
5. An isolated and purified DNA molecule of at least seven nucleotide bases which hybridizes to the DNA of claims 1, 2, or 3 under high stringency conditions.
6. An isolated and purified DNA molecule which encodes a turkey vasoactive intestinal peptide which hybridizes to a DNA sequence complementary to SEQ ID NO:5 under high stringency conditions.
7. A hybridization probe comprising an isolated and purified DNA molecule of at least seven nucleotide bases, which is detectably labeled or which binds to a detectable label, which DNA molecule hybridizes to the DNA molecule of claims 1, 2, or 3 under high stringency conditions.

8. An expression cassette comprising: a DNA molecule comprising a DNA segment encoding an avian vasoactive intestinal peptide operably linked to a promoter functional in a host cell.
9. The expression cassette according to claim 8, wherein the DNA segment encoding an avian vasoactive intestinal peptide encodes a turkey vasoactive intestinal peptide.
10. The expression cassette according to claim 8, wherein the DNA segment has SEQ ID NO:7.
11. The expression cassette according to claim 8, wherein the DNA segment has SEQ ID NO:2.

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1  CGACGGATTCCGGCTCCGTGGGGGCC  ATG  GAG  CAC  CAC  CGC  GGC  ACC  TCC  CCG  CTC  CTC  CTC  GGC  CTC  GGC  CTC
      10
Met  Glu  His  Arg  Gly  Thr  Ser  Pro  Leu  Leu  Leu  Ala  Leu
      20
Ala  Leu  Leu  Ser  Ala  Leu  Cys  Trp  Arg  Ala  Arg  Ala  Leu  Pro  Pro  Arg  Gly  Ala  Ala  Phe
      30
67  GCC  CTC  CTC  AGC  GCC  CTC  TGC  TGC  TGG  AGG  GCG  GCG  GCG  CCC  CCG  CGG  GGC  GGC  GCC  TTC
      40
Pro  Ala  Val  Pro  Arg  Leu  Gly  Asn  Arg  Leu  Pro  Phe  Asp  Ala  Ala  Ser  Glu  Ser  Asp  Arg
      50
127  CCT  GCT  GTG  CCG  CGA  CTG  GGA  AAC  AGA  CTG  CCC  TTT  GAT  GCA  GCC  AGT  GAA  TCT  GAC  CGC
      60
Ala  His  Gly  Ser  Leu  Lys  Ser  Glu  Ser  Asp  Ile  Leu  Gln  Asn  Thr  Leu  Pro  Glu  Asn  Glu
      70
187  GCC  CAT  GGG  TCC  CTA  AAG  TCT  GAA  TCA  GAC  ATT  TTG  CAG  AAC  ACA  CTA  CCT  GAA  AAT  GAG
      80
Lys  Phe  Tyr  Phe  Asp  Leu  Ser  Arg  Ile  Ile  Asp  Ser  Ser  Gln  Asp  Ser  Pro  Val  Lys  Arg
      90
247  AAA  TTC  TAT  TTT  GAT  TTG  TCC  AGA  ATT  ATT  GAT  AGC  TCC  CAG  GAC  AGT  CCT  GTC  AAA  CGC
      100
His  Ser  Asp  Ala  Val  Phe  Thr  Asp  Asn  Tyr  Ser  Arg  Phe  Arg  Lys  Gln  Met  Ala  Val  Lys
      110
307  CAC  TCT  GAT  GCT  GTC  TTC  ACT  TAC  AAT  TAC  AGC  CGC  TTT  CGA  AAG  CAA  ATG  GCT  GTG  AAG
      120
Lys  Tyr  Leu  Asn  Ser  Val  Leu  Thr  Gly  Lys  Arg  Ser  Gln  Glu  Glu  Leu  Asn  Pro  Ala  Lys
      130
367  AAA  TAC  TTA  AAC  TCA  GTT  TTA  ACT  GGA  AAA  CGA  AGC  CAG  GAA  GAG  TTA  AAT  CCA  GCC  AAA
      140
Leu  Arg  Asp  Glu  Ala  Glu  Ile  Leu  Glu  Pro  Ser  Phe  Ser  Glu  Asn  Tyr  Asp  Val  Ser
      150
427  CTT  GCA  GAT  GAA  GCA  GAA  ATT  CTT  GAA  CCT  TCC  TTT  TCA  GAA  AAC  TAT  GAT  GAT  GTT  TCT
      160
Val  Asp  Glu  Leu  Leu  Ser  His  Leu  Pro  Leu  Asp  Leu  END
487  GTA  GAT  GAA  CTG  CTG  AGC  CAC  CTC  CCA  TTG  GAC  CTC  TGA  AGGACACCTAGAAAACCTCTTCAACAAG
553  AACAAAGTTATTTTGGAGTCCACATAGTATTTCAAAGAGATGACTTTAGTCATCAAAATCTGAACAAATATGTTGTGAAG
632  TGAAGTTGTGATATATTATTCTTATGTAATAAAGTTGATATTGTAATACTGTTCTAGAGTCTCTACT
711  GAAAGCTGTACATATGATGCCAGTTAAACAAATGAGAGTCTGTAGTCCATATGCTGTAAATCCTTTACTTCAATAA
790  ATTCAATTGAAAAATGAAAAAAA

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FIG. 1

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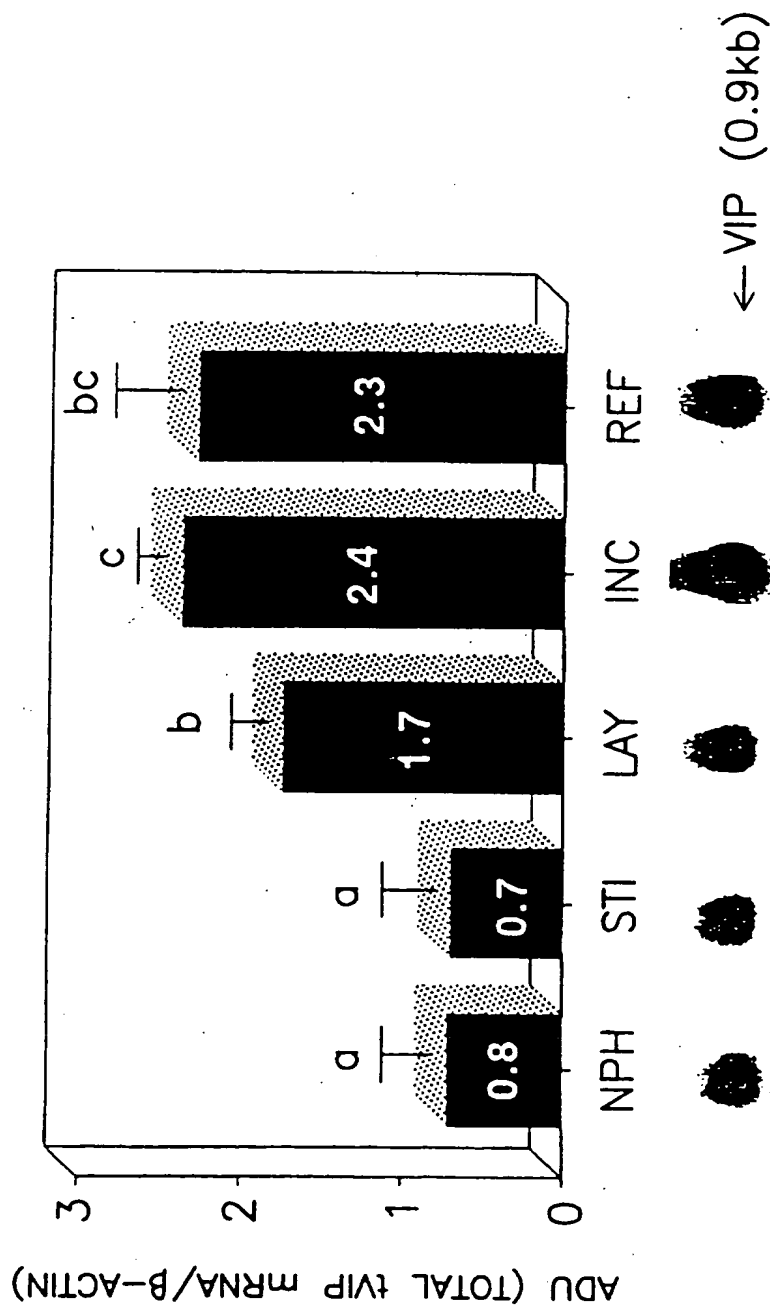


FIG. 2

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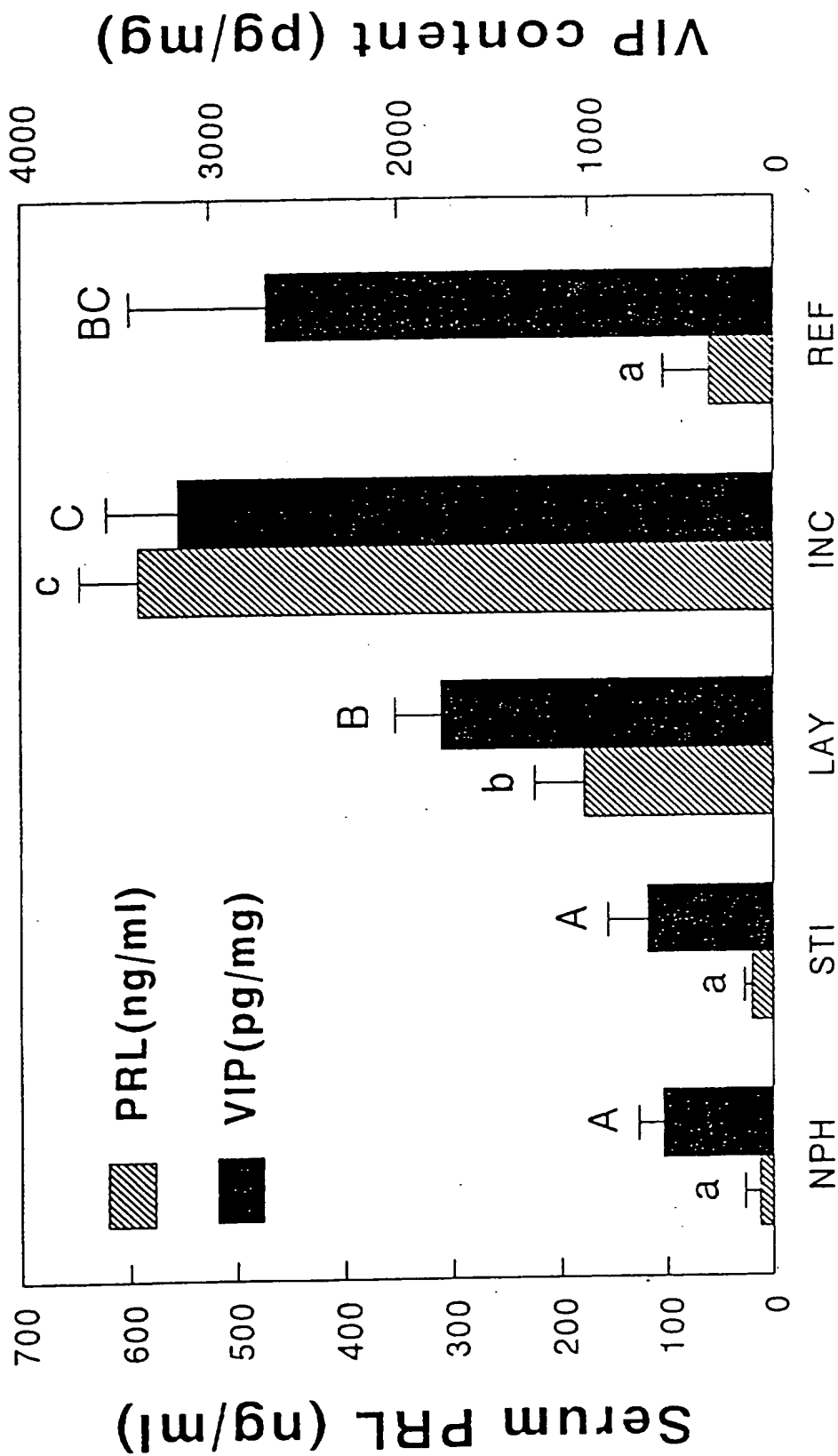


FIG. 3

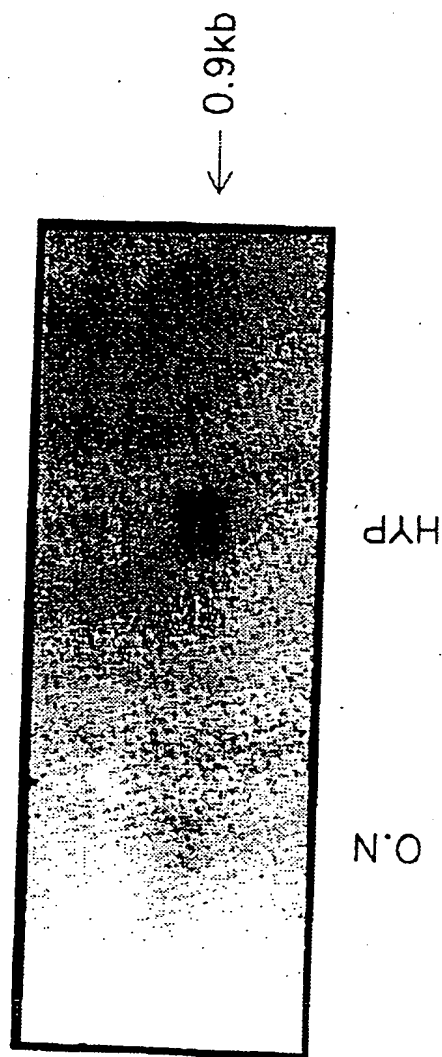


FIG. 4



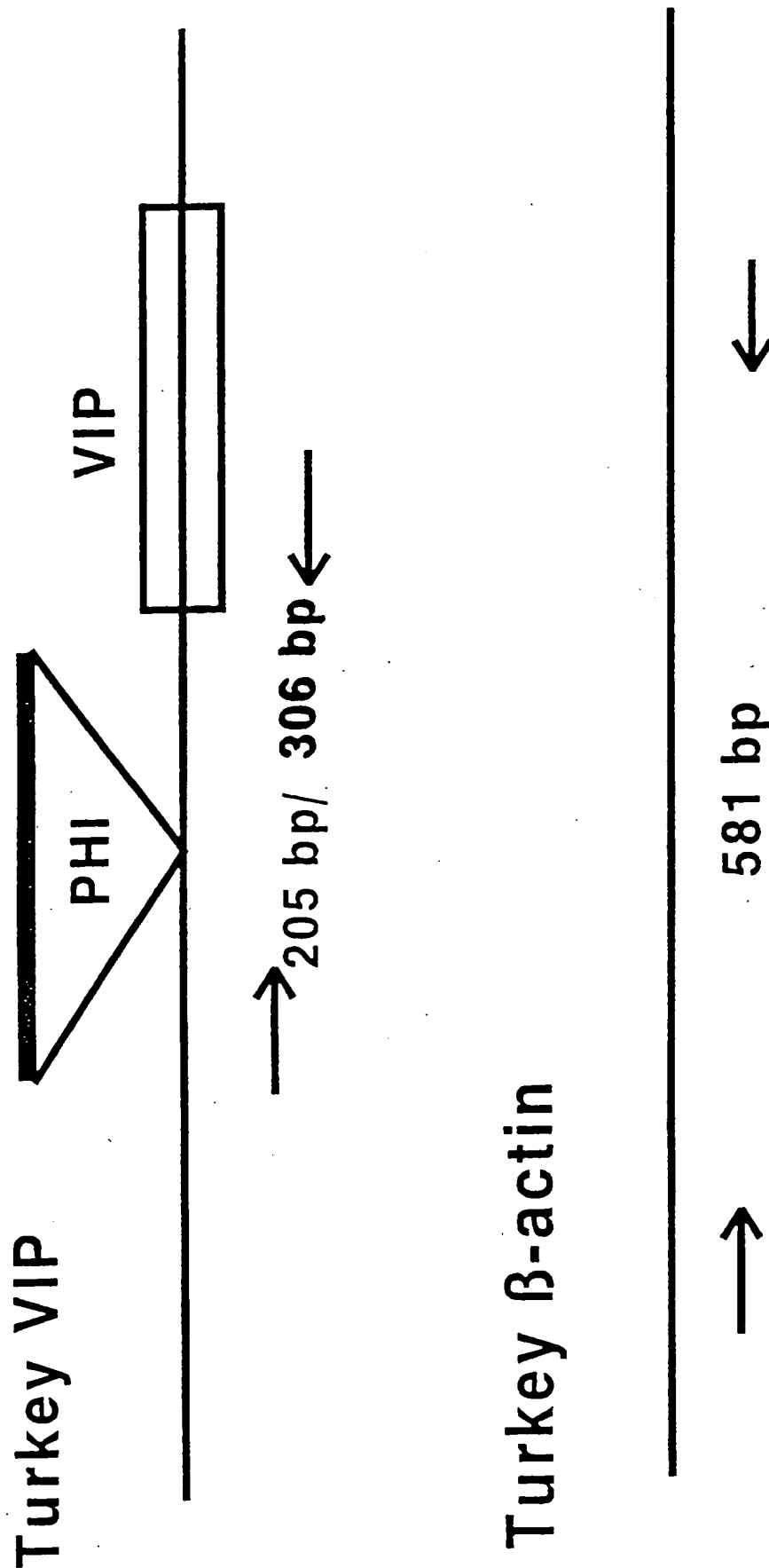


FIG. 5

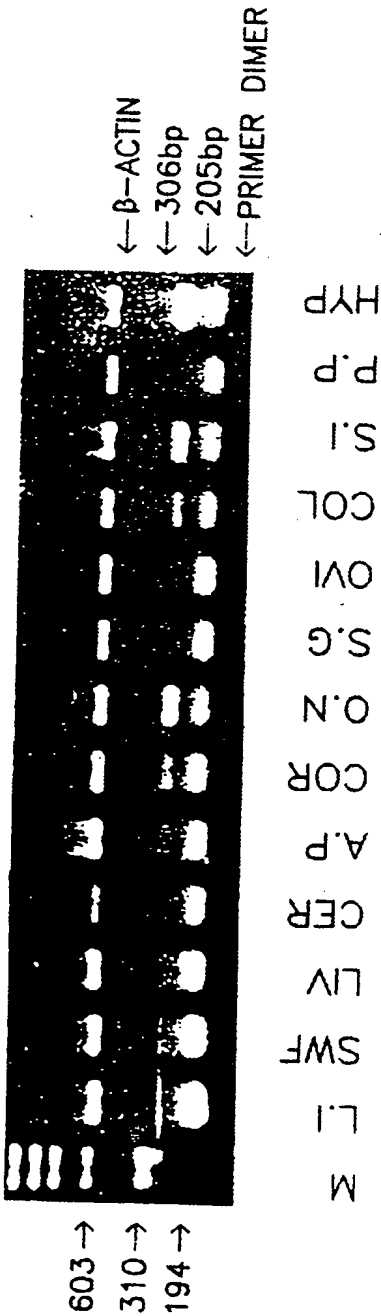


FIG. 6

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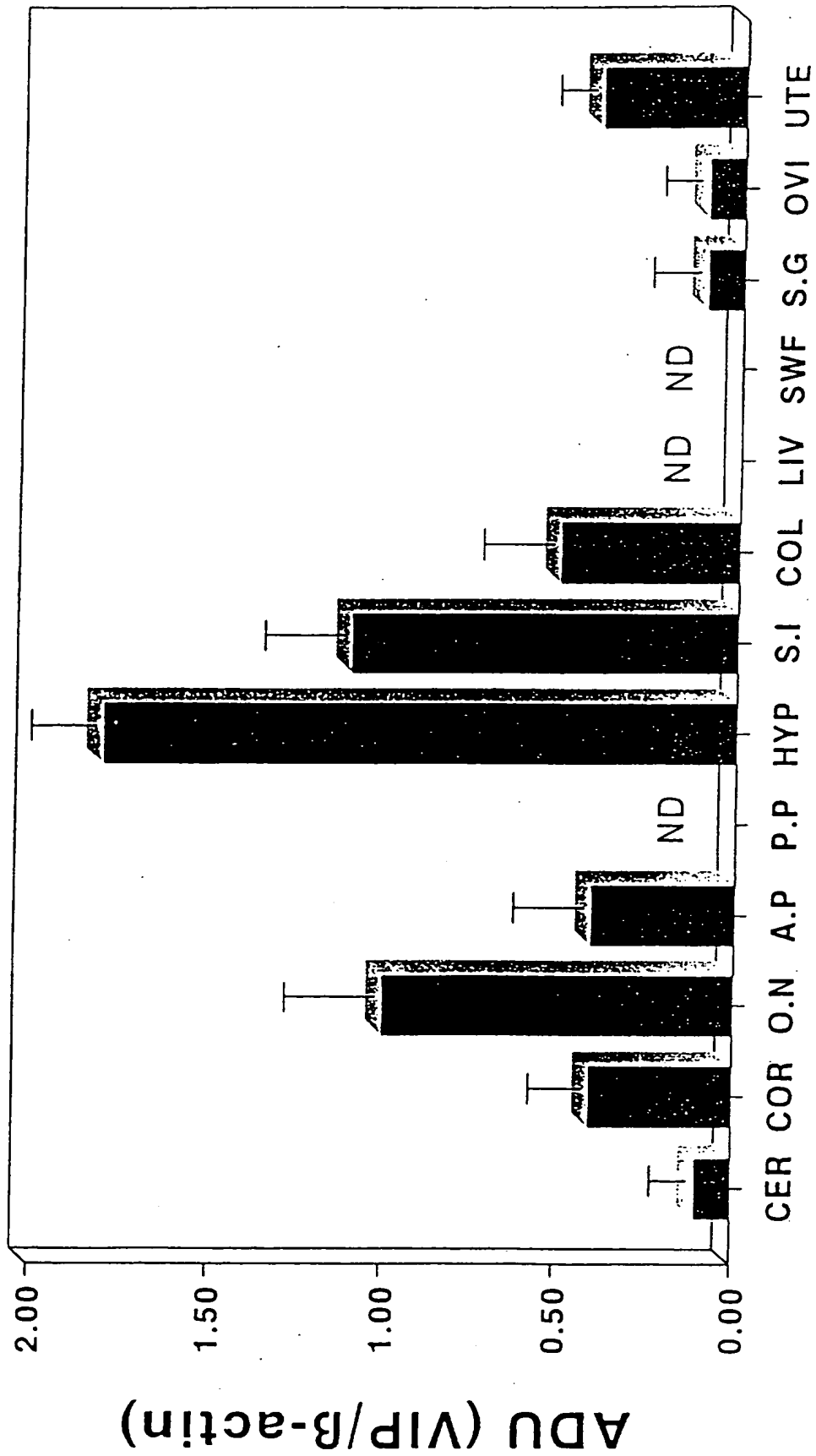


FIG. 7

AGA AAT GCA AGG CAT GCT GAT GGA ATT TTC ACC ACT  
 Arg Asn Ala Arg His Ala Asp Gly Ile Phe Thr Thr

GTA TAC AGC CAT CTT TTG GCT AAA CTC GCT GTG AAG  
 Val Tyr Ser His Leu Leu Ala Lys Leu Ala Val Lys

AGA TAT CTG CAT TCG CTT ATT AGA AAA AGA GTT  
 Arg Tyr Leu His Ser Leu Ile Arg Lys Arg Val

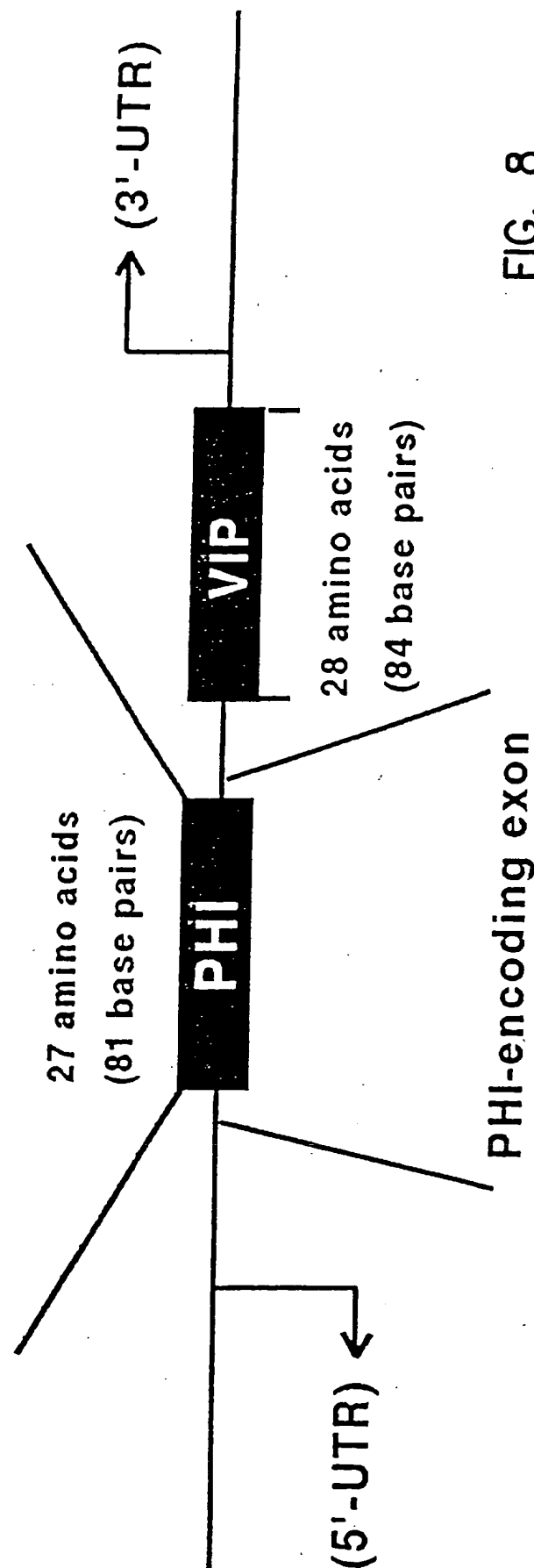


FIG. 8

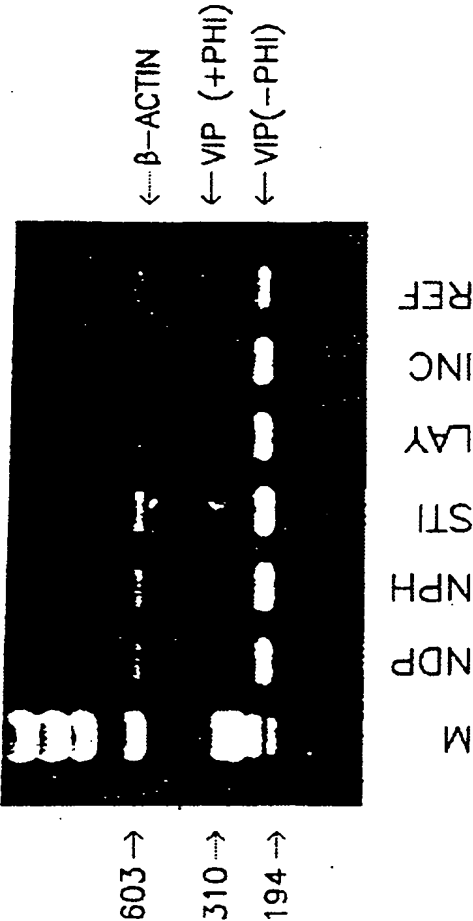
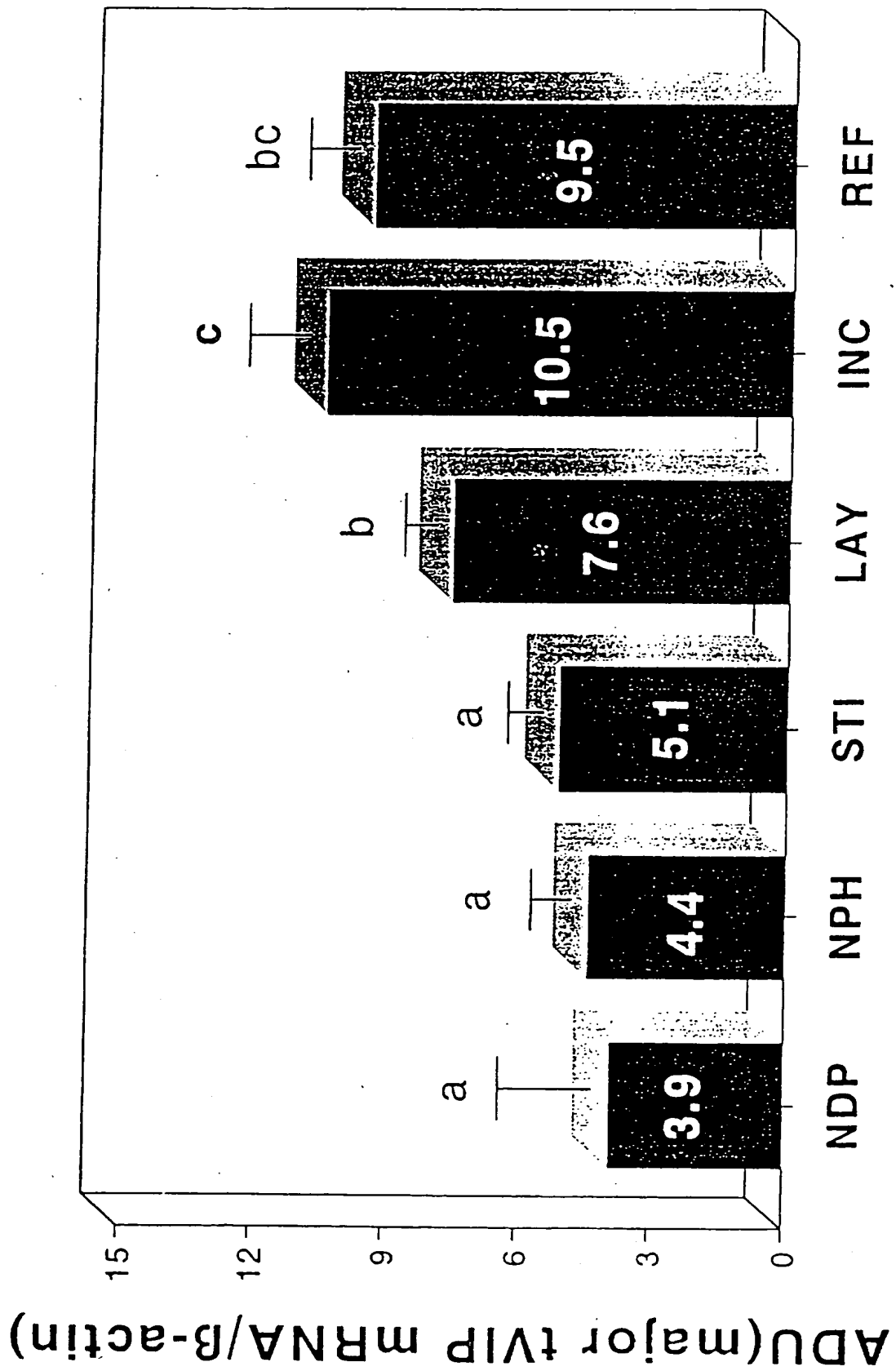


FIG. 9



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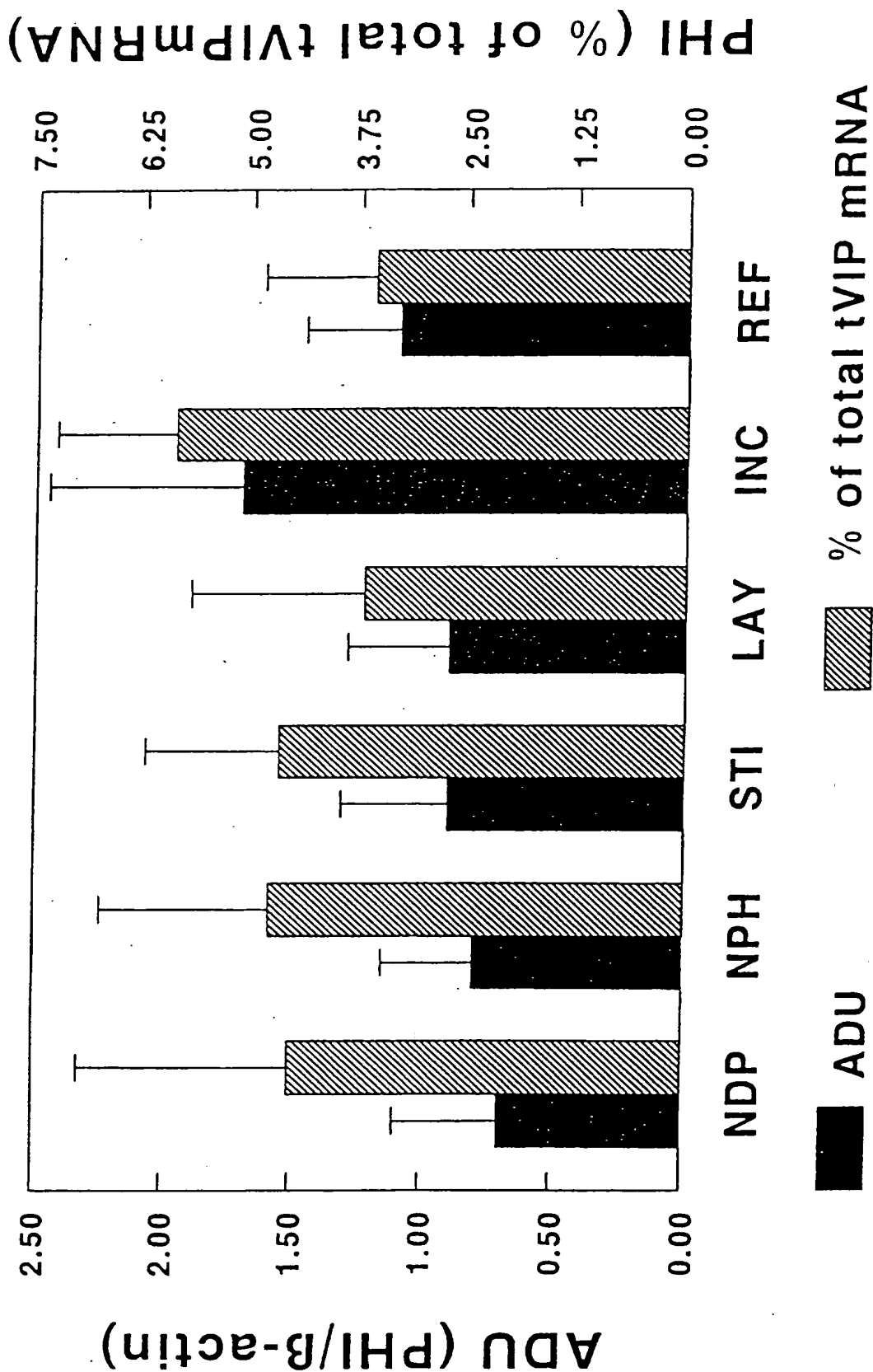


FIG. 11

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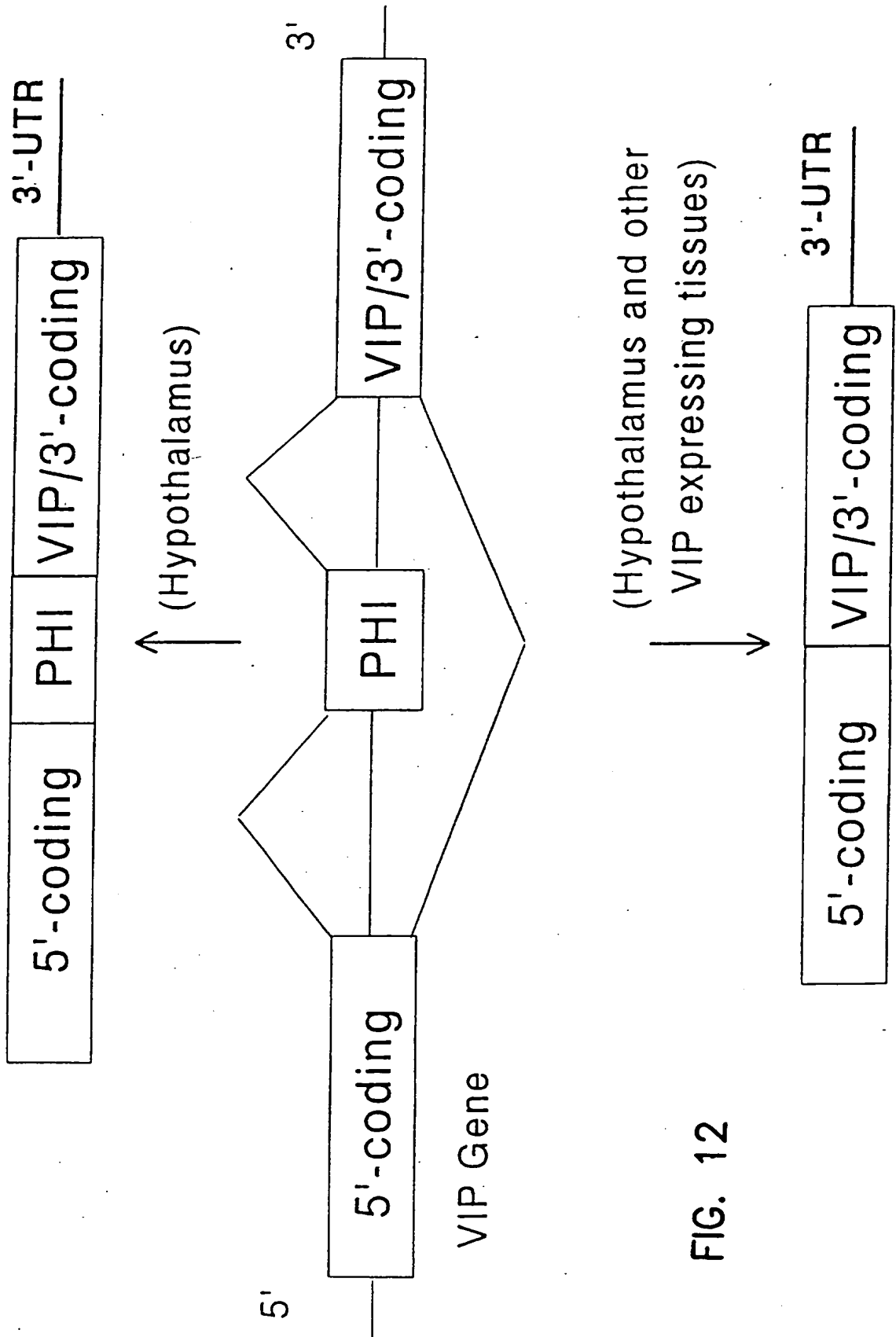


FIG. 12



# INTERNATIONAL SEARCH REPORT

National Application No.

PCT/US 95/10075

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N15/16

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE STRAND AN : emvrt : gg09350, 31 May 1994 see sequence & UNPUBLISHED, FARLIN ET AL. 'Structure of chicken vasoactive intestinal peptide cDNA' see DNA sequence and title ---	1,2,4,5, 7
P,X	ENDOCRINOLOGY, vol. 136, no. 6, June 1995 pages 2602-2610, YOU ET AL. 'Tissue-specific alternative splicing of turkey preprovasoactive intestinal peptide messenger ribonucleic acid, its regulation, and correlation with prolactin secretion' see the whole document --- -/--	1-11

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- '&' document member of the same patent family

Date of the actual completion of the international search

23 November 1995

Date of mailing of the international search report

21.12.95

Name and mailing address of the ISA

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Fax (+ 31-70) 340-3016

Authorized officer

Gac, G

## INTERNATIONAL SEARCH REPORT

International Application No.  
/US 95/10075

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 08616 (REGENTS OF THE UNIVERSITY OF MINNESOTA) 28 April 1994 see page 4 paragraphs 1 and 3 see page 5 second paragraph ---	1-11
A	GEN. COMP. ENDOCRINOL., vol. 87, no. 3, September 1992 pages 481-493, MAURO ET AL. 'Effects of reproductive status, ovariectomy and photoperiod on Vasoactive intestinal peptide in the female turkey' see abstract ---	1-11
A	US,A,4 016 258 (SAID ET AL.) 5 April 1977 see the whole document -----	1-11

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
Please see annex!
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/

Through the careful reading of the application, a lot of serious inconsistencies and contradictions in the numbering of the sequences between the description and the sequence listing have been detected, rendering a meaningful search hardly possible. However, an extensive and accurate comparison of the above-mentioned sequences, coupled to the general knowledge of a person skilled in the art, allowed the reconstitution of the application in a way which would render it both intelligible/comprehensible and meaningfully searchable. Therefore, in light of the sequences provided in Figures 1 and 8, of page 71.7-11, page 81.25-26 and page 171.25-30, in comparison with the sequences provided in the sequence listing (seq. no. 12-14 do not exist, some sequences disclosed as "DNA" are (in the seq. listing) proteins, many do not correspond to the description), it appears that the whole application should be redrafted (concerning seq. numbering) according to the following scheme and with respect to the numbering of the sequence listing:

- Sequence no.1 of description = seq.1 of seq.listing = mature tVIP.
  - Sequence no.2 of description = seq.2 of seq.listing= DNA for mature tVIP..
  - Sequence no.5 of description is the antisense nucleotide to seq.no.2. and corresponds (is identical) to seq.no.3 of seq.listing.
  - Sequence no.7 of description = seq.5 of seq.listing= DNA coding for the prepro-tVIP (native protein).
  - Sequence 8 of description = seq.4 of seq.listing= prepro-tVIP native protein.
  - Sequence 9 of description = seq.6 of seq.listing= DNA coding for PHI-encoding exon.
  - Sequence 10 of description= seq.7 of seq.listing= PHI protein.
  - Sequence 11 to 14 of description do not exist in the sequence listing under these seq.numbers but correspond to sequences 8 to 11 of the seq.listing (seq. 8 and 9 = VIP-specific primers, seq.10 -11 =  $\beta$ -actin specific primers).
- The search was performed according to the above-mentioned seq. numbering correspondence (claims seq.nr interpreted accordingly).

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Initial Application No

PCT/US 95/10075

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9408616	28-04-94	AU-B- 5163693 CA-A- 2144950 EP-A- 0661997	09-05-94 28-04-94 12-07-95
US-A-4016258	05-04-77	NONE	

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